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Semen as a source of *Mycoplasma bovis* mastitis in dairy herds

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ABSTRACT

Mycoplasma bovis infections are responsible for substantial economic losses in the cattle industry, have significant welfare effects and increase antibiotic use. The pathogen is often introduced into naive herds through healthy carrier animals.

In countries with a low prevalence of *M. bovis*, transmission from less common sources can be better explored as the pathogen has limited circulation compared to high prevalence populations. In this study, we describe how *M. bovis* was introduced into two closed and adequately biosecure dairy herds through the use of contaminated semen during artificial insemination (AI), leading to mastitis outbreak in both herds. Epidemiological analysis did not reveal an infection source other than semen. In both farms the primary clinical cases were *M. bovis* mastitis in cows inseminated with the semen of the same bull four weeks before the onset of the disease. One semen straw derived from the semen tank on the farm and other semen lots of this bull were positive for *M. bovis*. In contrast, semen samples were negative from other bulls that had been used for insemination in previous or later oestrus to those cows with *M. bovis* mastitis. Furthermore, cgMLST of *M. bovis* isolates supported the epidemiological results. To our knowledge this is the first study describing the introduction of *M. bovis* infection into a naive dairy herd via processed semen. The antibiotics used in semen extenders should be re-evaluated in order to provide farms with *M. bovis*-free semen or tested *M. bovis*-free semen should be available.

1. Introduction

Mycoplasma bovis infection causes substantial economic losses and welfare effects in the cattle industry (Nicholas and Ayling, 2003) and increases the use of antibiotics. The infection presents a variety of signs, the most common being respiratory disease, mastitis and joint infections. (Byrne et al. 2001). The prevalence of *M. bovis* varies in different countries and areas.

Animal contact is the main source of *M. bovis* infection. The major risks for infection are related to animal movement, animal purchase and animal fairs (Amram et al. 2013; Aebi et al. 2015). Other well known risks include artificial insemination (AI) (Wrathall et al. 2007), embryo transfer (Bielanski et al. 2000), contaminated equipment and environment (Piccinini et al. 2015), airborne transmission (Jasper et al., 1974) and contact with infected people (Madoff et al. 1979) or other animal species (Dyer et al. 2004; Ongor et al. 2008; Spergser et al., 2013).

A reproductive challenge study was able to induce *M. bovis* intramammary infection in nine cows following intra-amniotic or intra-arterial inoculation (Ruben, 1980). *Mycoplasma bovis* has been isolated in commercial semen (Amram et al. 2013), with *M. bovis* positive semen reported to cause alterations in the fertilization process leading to infertility (Eaglesome and Garcia, 1990) and pathological alterations in the reproductive organs after experimental intrauterine challenge (Hartman et al. 1964).

In countries with a low prevalence of *M. bovis*, transmission from less common sources can be better explored as the pathogen has limited circulation compared to high prevalence populations. *Mycoplasma bovis* was detected for the first time in Finland at the end of 2012 and has since spread among dairy herds. It has since been monitored because it is of national interest to control the infection. In this study, we describe how *M. bovis* was introduced into two closed and adequately biosecure dairy herds through the use of contaminated semen during artificial insemination (AI), leading to mastitis outbreak in both herds. As far as we know, our study is the first to demonstrate that semen used in AI can

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be the initial source of *M. bovis* infection on a farm.

2. Material and methods

2.1. Surveillance of M. bovis in Finland

M. bovis was detected in Finland for the first time in November 2012 in deep nasopharyngeal samples from pneumonic calves in a calf rearing farm. The calves in this rearing unit originate from several dairy farms. The infection was indicated to be recent. *Mycoplasma bovis* was not detected in a research project on bovine respiratory disease in calf-rearing units in 2002–2003 (Autio et al. 2007). Since 2003, suspected cases, such as indicated by respiratory disease and aborted fetuses, have been examined for *M. bovis* using culture and PCR. Mastitis pathogen testing of individual milk samples from clinical and subclinical mastitis has a long tradition in Finland. Roughly 150 000 samples were tested in 2015 (285 000 cows in Finland in 2015). Since early 2012, almost all mastitis diagnostic laboratories use multiplex PCR tests that target also *M. bovis* (Thermo Scientific PathoProof Mastitis Complete-16 assay, Thermo Fisher Scientific Ltd.). Practicing veterinarians have to report all *M. bovis* cases monthly to animal disease control authorities. During 2012–2015, altogether 20 Finnish dairy farms were infected with *M. bovis*, which represented 0.26% of all 7 600 Finnish dairy farms in 2015.

Since 2013 the organization Animal Health ETT maintains a voluntary *M. bovis* control program for cattle farms. The program includes regular veterinary health care visits, restricted animal movement, surveillance of signs and laboratory examination of subclinical and clinical mastitis samples with PCR and nasal swabs from calves. The data are entered into a centralized health care register (Naseva) for cattle herds. The register includes production data, health records and veterinary treatments. A total of 75% of all dairy farms belonged to the health register in 2017 (Animal Health ETT, personal communication).

2.2. Farms in the study

Farmers from 20 newly infected *M. bovis* dairy farms were interviewed and their herds were sampled. Epidemiological data were collected to assess the infection source. In two concurrently infected farms the epidemiological data did not suggest a typical infection source and the farms (X and Y) were investigated in more detail.

2.3. Herd data collection

Herd data were collected using the centralized health care register and by use of a questionnaire. A project veterinarian visited both farms twice. Information gathered from the registers included the number of cattle and cows, milk yield, mortality rate, treatments, dates of *M. bovis* mastitis, the number of cattle slaughtered, meat rejected, the number of milk samples, numbers of purchased and imported cattle, milk-recording system and laboratory results of nasal swab analyses related to the *M. bovis* control program.

Information gathered from the farmers included the housing type, milking system, bedding material, cattle movements, contract heifer rearing unit, imported or domestic embryos, health care visits, protective clothing, loading area, corporate truck, equipment of the hoof trimmer and AMS (automatic milking system) maintenance, vermin and bird control, the number of cattle slaughtered, meat rejected and participation in the *M. bovis* control program.

Distance to the closest cattle farm was determined from a national register. Insemination data (dates, bulls and lots) was gathered from the Finnish Animal Breeding Association (FABA).

2.4. Samples

We examined 98 deep-frozen semen straws, representing 32 lots from ten bulls (A–J) used in AI in herds X and Y. Four semen straws were taken from the liquid nitrogen tank from farm X, and the rest were obtained from the breeding company. The straws examined belonged either to the same lot used in the herds or the closest lot available.

Semen from bull A was collected and handled at a semen collection center and was examined according to OIE requirements. Collection of semen started at the age of 11 months. Based on the results of semen samples, conjunctival swabs, nasal swabs, preputial swabs (Transsystem, Copan Brescia, Italy), pre-ejaculate and semen samples were taken from the bull A at the age of 2 years 4 months. Pre-ejaculate and semen were placed in F-broth for mycoplasma culture.

A total of 15 and 20 calves were sampled on farms X and Y, respectively, 4 to 5 weeks after the primary infection. Nasal swabs (Transsystem) were collected from calves 1 week to 6 months old.

2.5. Culture of M. bovis

The straws (n = 58) were thawed in 37 °C water bath. Semen from the straw was inoculated in a tube containing 2.7 ml F broth prepared according to Bölske (1988). Inoculated F broth was diluted 10-fold up to 10^{-6} and incubated at 37 °C for 14 days. The growth and color change were monitored every other day, and samples suspected of containing mycoplasma were subcultured onto F-medium plates (Bölske, 1988). Plates were incubated at 5% CO_2, 37 °C for seven days, and inspected every second day under the microscope for mycoplasma growth. *Mycoplasma bovis* was identified using PCR.

2.6. DNA extraction and Mycoplasma bovis real time PCR

DNA was extracted from semen straws (n = 40) using a QIAamp Mini Kit (Qiagen, Hilden, German), following the manufacturer's protocol for blood and body fluids. DNA was eluted from the spin column with 50 µl of water. The protocols described by Sachse et al. (2010) were used for nasal swabs and broth cultures.

Semen, nasal swabs and broth cultures were examined using real time PCR targeting the *opp*D gene of *M. bovis* (CFX96 Touch Real-Time PCR Detection System, Bio-Rad Laboratories, CA, USA) as described previously (Sachse et al. 2010). Commercially available plasmid pUC19 was used as the internal amplification control according to Fricker et al. (2007), except that BHQ1 was used instead of TAMRA in the probe.

2.7. Whole genome sequencing

Whole Genome Sequencing (WGS) was used to compare genomes. A total of 11 *M. bovis* isolates were included in the study (Table 1): the isolate 11911 from the first mastitis case on farm X, the isolate 13775 from the first mastitis case on farm Y, the isolate 198 from semen lot 3 from bull A, seven isolates obtained from diseased cattle in Finland during 2012–2015, and one isolate from a pneumonic calf in Estonia. The isolates were selected to represent various herd types, clinical presentations and geographical distribution within Finland, including isolates from the first two *M. bovis* cases in Finland.

All isolates were purified three times before freezing and were stored in F-broth medium (Bölske, 1988) at −80 °C. For DNA extraction, the isolates were grown in 50 ml F-broth in closed tubes at 37 °C for 90 h. The bacteria were spun down at +4 °C, 19,800 g, for 30 min. The pellets were washed with sterile PBS and spun down again. The pellets were resuspended in 180 µl of sterile PBS. DNA was extracted using a QIAamp Mini Kit, following the manufacturer's protocol for blood and body fluids. DNA concentration was measured (Qubit dsDNA BR assay system, Invitrogen, Carlsdab, CA) and quality assessed in 0.8% gel electrophoresis.

WGS was done at the Danish Technical University, Department for Biotechnology and Biomedicine, Lyngby, Denmark. A Nextera XT kit (Illumina, San Diego, CA) was used according to the manufacturer's instructions to prepare the libraries for WGS. An Illumina MiSeq

Table 1
Description of *Mycoplasma bovis* isolates sequenced in this study.

Isolate ID	Farm type/country	Year of isolation	Clinical presentation / sample type
10419	calf rearing / FI	2012	respiratory disease / nasopharyngeal swab
10585	dairy / FI	2012	mastitis / milk
9578	dairy / FI	2013	mastitis / milk
3738	beef cattle / FI	2013	respiratory disease / lung
7818	dairy / FI	2014	mastitis / milk
6414	dairy / FI	2014	arthritis / joint fluid
16981	calf rearing / FI	2014	respiratory disease / nasopharyngeal swab
119111[a]	dairy / FI	2015	mastitis / milk
198[b]	bull station / DK	2015	clinically healthy / semen
13775[c]	dairy / FI	2015	mastitis / milk
537	dairy / EE	2011	respiratory disease / tracheobronchial lavage

FI = Finland, DK = Denmark, EE = Estonia.
[a] Farm X.
[b] Bull A used in Farm X and Y.
[c] Farm Y.

(Illumina) platform with 300 base paired-end sequencing was used.

2.8. Core genome MLST analysis

De novo assembly of the sequencing reads was performed using Velvet assembler version 1.1.04 (Zerbino and Birney, 2008) of Ridom SeqSphere+ (Ridom GmbH, Münster, Germany) software (Jünemann et al. 2013). Automated k-mer and coverage cutoff optimizations were performed for each assembly.

The BLAST-based target definer function (Altschul et al. 1990) of the Ridom SeqSphere + software was operated to identify 527 core genome and 168 accessory genome target loci from the reference strain NC_014760.1 (*M. bovis* PG 45) and seven complete query genomes obtained from GenBank (NZ_CP005933.1, NC_018077.1, NC_015725.1, NZ_CP011348.1, NZ_CP007589.1, NZ_CP007590.1 and NZ_CP007591.1). A minimum spanning tree (MST) within the Ridom SeqSphere + software was constructed to visualize the results.

3. Results

3.1. Farm data

Both farms were loose house dairy farms with good farming practice (Table 2) and good or excellent biosecurity (Table 3). In both farms milk samples from clinical mastitis, high somatic cell count (SCC) quarters and before drying off, were regularly monitored for pathogens, including *M. bovis*. Farm Y belonged to the *M. bovis* control program and had been sampled twice for *M. bovis* with negative results in 2013 and 2014. Our epidemiological analysis did not find any significant route of infection other than semen (Table 3).

Mycoplasma bovis was diagnosed for the first time in both farms from quarter milk samples with clinical mastitis. Starting in November 2015, farm X had four *M. bovis* mastitis cases within five weeks (Fig. 1). Farm Y had three *M. bovis* mastitis cases within four weeks, the first one in December 2015 (Fig. 2). There were no previous signs at either farm and the levels of medical treatment were normal. *Mycoplasma bovis* was detected from calves in both farms 4–5 weeks after the primary case. Altogether four cows in these herds had been inseminated with semen from which *M. bovis* was isolated. All but one of the cows developed *M. bovis* mastitis.

3.2. Mycoplasma bovis in semen and bull samples

Altogether ten bulls (A–J) were used to inseminate cows that developed *M. bovis* mastitis. One of them (bull A) had *M. bovis*-positive semen lots (Table 4). Of the 22 examined semen lots from bull A, lots 4,

Table 2
Description of farms X and Y in 2015.

Farm description	Farm X	Farm Y
No. of animals	139	126
No. of cows	60	61
Housing type	Loose house	Loose house
Milking system	AMS[a]	AMS
Ave. milk yield in the herd (kg)	9561	9984
Bedding material		
Calves	Peat moss	Straw
Cows	Sawdust	Sawdust
Veterinary health care visits in 2015	6	12
Mortality rate in 2015	0.7 %	3.25 %
Related signs treated in 2015 (no. of cases before primary case)		
Mastitis other than *M. bovis*	7	8
Arthritis	1	0
Respiratory signs with young stock	1	3
No. of animals slaughtered / meat rejected	28 / 7 kg	13 / 0 kg
Finnish milk-recording system	Yes	Yes
Centralized health care register of cattle	Yes	Yes
Distance to closest cattle farm	1.2–5 km, 5 farms	1.6–5 km, 3 farms
No. of milk samples examined for *M. bovis* in 2015 before primary case	41	200

[a] Automatic milking system.

Table 3
External biosecurity activities related to *M. bovis* introduction to the farms.

External biosecurity risk factors	Farm X	Farm Y
Last purchase of cattle	2011	2003
Cattle movements (e.g. animal fairs)	None	None
Contract heifer rearing unit	No	No
Imported cattle	None	None
Imported embryos[a]	None	None
Domestic embryos[a]	Yes	Yes
Protective clothing and footwear	Yes[b]	Yes
Separate loading area	No	Yes
Uses a corporate truck to move own animals	No	No
M. bovis control program	No	Yes
Distance to known *M. bovis*-positive farm	87 km	87 km
Hoof trimmer, equipment properly cleaned	–[c]	Yes
AMS[d] maintenance, equipment properly cleaned	Yes	Yes
Vermin and bird control	Yes	Yes

[a] Within three years before the primary case.
[b] Before 2011, the driver of slaughter animals and calves has rarely been inside the barn with own gear and veterinarian has used own protective gear.
[c] No corporate trimmer used.
[d] Automatic milking system.

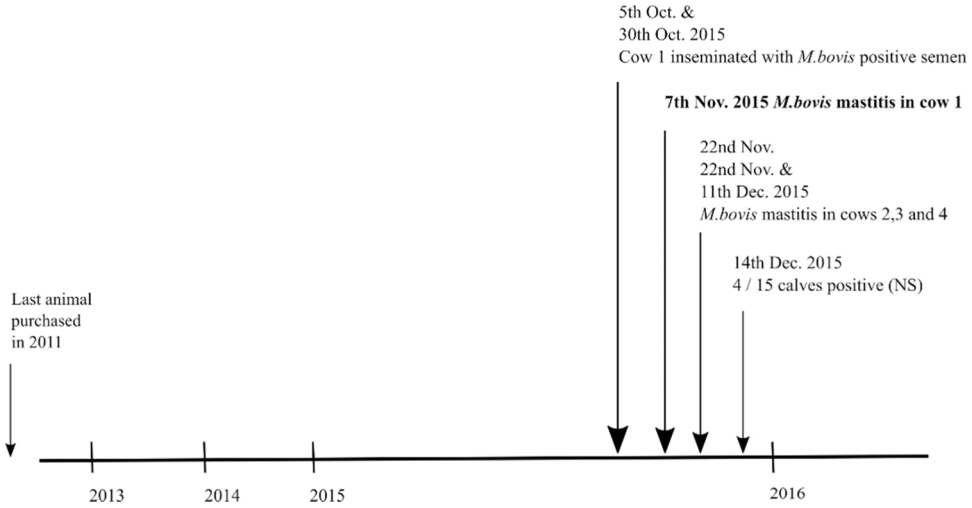

Fig. 1. Course in *M. bovis* infection in herd X. NS, nasal swabs of calves, positive/total examined for *M. bovis*.

6 and 12 were positive following PCR analysis and lots 3 and 4 were positive in culture (Table 5). All mucosal swabs as well as pre-ejaculate and sperm samples taken from bull A at the age of 2 years were negative for *M. bovis* following PCR or culture.

3.3. Core genome MLST analysis

The core-genome multilocus sequence typing (cgMLST) schema targets covered 58.2% of the reference genome. From the 11 isolates selected for this study, 589 cgMLST allele-called targets were extracted and compared with each other. Except for the Estonian strain 537, all other isolates clustered together within a 4–24 allele difference. The mastitis strains from farms X and Y and the bull A semen strain had allele differences of 4 and 8, respectively, and clustered together (Fig. 3).

4. Discussion

To our knowledge this is the first study to describe the introduction of *M. bovis* infection into a naive herd via processed semen. Epidemiological analysis did not establish any source other than *M. bovis*-contaminated processed semen used for insemination. It was possible to identify this rare incidence in a country where both *M. bovis* prevalence and cattle density are low. In both farms the primary clinical cases were mastitis caused by *M. bovis* in cows that had been inseminated with the semen from the same bull four weeks before the onset of the disease. The semen originating from the liquid nitrogen tank from farm X, as well as some other lots of semen from this bull, were positive for *M. bovis*. In contrast, semen samples were negative from other bulls that had been used for insemination in previous or later oestrus to those cows with *M. bovis* mastitis. Furthermore, cgMLST supported the epidemiological results. There was a difference of four alleles between isolate 198 (semen from bull A) and isolate 11911 (farm X, mastitis) and eight alleles between isolates 11911 and 13775 (farm Y, mastitis). These isolates clustered separately from the other Finnish isolates, although there was limited diversity among the Finnish isolates. The results strongly support the finding that semen positive for *M. bovis* used in insemination was the source of the *M. bovis* infection.

From all the farm data, we concluded that both farms X and Y were free of *M. bovis* infection before the primary mastitis cases. Farm Y had reached the low risk level in the voluntary *M. bovis* control program. Neither farm had purchased live animals during the last four years, nor did they use contract heifer rearing or attend cattle shows with their animals. Both farms were situated in an area of low cattle density. Mastitis samples had been regularly examined for *M. bovis* using PCR with negative results since February 2012.

The most common means of contracting *M. bovis* by a naive herd is through purchase of a clinically healthy animal shedding the agent (Gonzalez et al. 1992; Aebi et al. 2015). Other possible sources include airborne transmission and fomites. There is no well-founded evidence for airborne transmission of *M. bovis*. Soehnlen et al. (2012) analyzed *M. bovis* content of the air of veal calf barns monthly and no *M. bovis* was detected, although 90.5% of the calves were colonized nasally and 40% bronchially. Only one previous report exists of *M. bovis* being

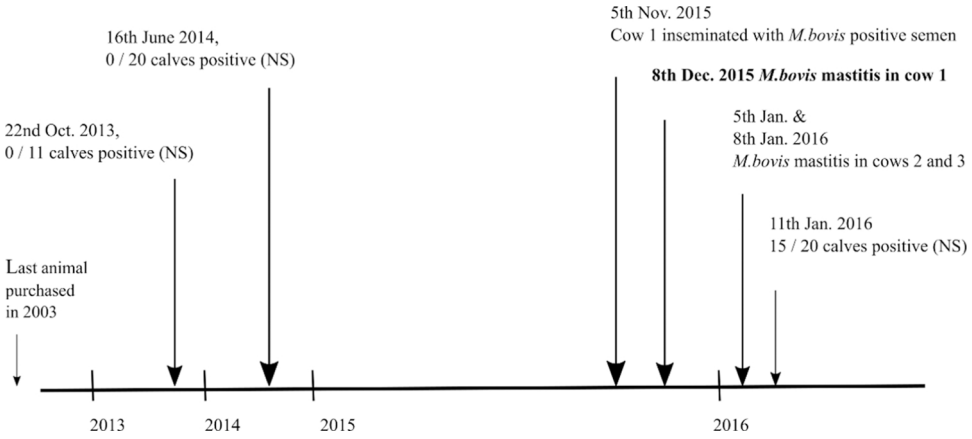

Fig. 2. Course in *M. bovis* infection in herd Y. NS, nasal swabs of calves, positive/total examined for *M. bovis*.

Table 4
Occurrence of *M. bovis* mastitis in farms where *M. bovis* contaminated semen of bull A was used.

Farm	Cow	*M. bovis* mastitis diagnosed (PCR)	Insemination date	Insemination No.	Bull used	*M. bovis* status of the semen
X	1		5.10.2015	1	A	positive
X	1	7.11.2015	30.10.2015	2	A	positive
X	2		26.8.2015	1	B	negative[b]
X	2		2.7.2015	2	B	negative
X	2		17.9.2015	3	C	negative
X	2		8.10.2015	4	A	positive
X	2	22.11.2015	1.11.2015	5	D	negative
X	3	22.11.2015	22.8.2015	1	E	negative[b]
X	4		8.10.2015	1	F	negative[b]
X	4		28.10.2015	2	F	negative
X	4	11.12.2015	30.10.2015	3	F	negative[b]
Y	5		20.9.2015	1	G	negative[b]
Y	5		23.9.2015	2	G	negative[b]
Y	5		13.10.2015	3	G	negative[b]
Y	5		5.11.2015	4	A	positive
Y	5	8.12.2015	29.11.2015	5	H	negative
Y	6		23.11.2015[a]	1		
Y	6	5.1.2016	29.12.2015	2	I	negative
Y	7		8.11.2015	1	F	negative
Y	7	8.1.2016	29.12.2015	2	J	negative

[a] Embryo transfer.
[b] Result from closest lot available of the lot used in AI.

Table 5
Detection of *M. bovis* by PCR and culture of the semen of bull A.

Lot number	Semen collection date	No of straws examined altogether from a lot	No of PCR positive/ tot. number examined	ct values	No of culture positive/ total number examined
1	20.4.2015	2	0/1	nd[b]	0/1
2	4.5.2015	2	0/1	nd	0/1
3	11.5.2015	5	0/2	nd	2[c]/3
4	20.5.2015	4	1/2	35.11	2[c]/2
5	27.5.2015	2	0/1	nd	0/1
6	8.6.2015	10	2/3	36.6/35.4	0/7
7	15.6.2015	2	0/1	nd	0/1
8	17.6.2015	3	0/1	nd	0/2
9	na[a]				
10	22.6.2015	2	0/1	nd	0/1
11	26.6.2015	2	0/1	nd	0/1
12	1.7.2015	10	1/3	37.5	0/7
13	3.7.2015	2	0/1	nd	0/1
14	6.7.2015	2	0/1	nd	0/1
15	20.7.2015	4	0/2	nd	0/2
16	29.7.2015	9	0/2	nd	0/7
17	31.7.2015	2	0/1	nd	0/1
18	26.8.2015	2	0/1	nd	0/1
19	31.8.2015	2	0/1	nd	0/1
20	2.9.2015	2	0/1	nd	0/1
21	9.9.2015	2	0/1	nd	0/1
22	na				
23	18.9.2015	2	0/1	nd	0/1
24	23.9.2015	2	0/1	nd	0/1

[a] Not available.
[b] Not detected.
[c] *M. bovis* growth only in 10^{-3} dilution.

successfully cultivated from the air by leaving an agar plate open in the barn housing calves with *M. bovis* associated disease (Jasper et al., 1974). Otake et al. (2010) describes airborne transmission of *Mycoplasma hyopneumoniae* from a population of growing pigs with experimental infection. *Mycoplasma hyopneumoniae* was recovered in 5.3% of air samples taken within 3.5–9.2 km of the source, and was shown to retain infectiousness. This suggests that during an outbreak under favorable weather conditions, airborne transmission might be possible. Barn ventilation, stocking density and pen design influence airborne transmission of mycoplasmas. Although environment and management differ between cattle and pig farms, results from *M. hyopneumoniae* air transmission studies might be applicable to *M. bovis*. In this study airborne transmission was most unlikely because the closest known *M. bovis*-positive farms were far apart (87 km).

Experimental inoculation of bulls´ preputium or urethra with *M. bovis* can produce ascending infection of the testes and shedding of *M. bovis* into semen (Kreusel et al. 1989). Different mycoplasmas can colonize the prepuce and distal urethra of normal AI bulls and can also be isolated from semen (Doig, 1981; Onoviran et al. 1975; Parker et al. 2017). In our study, bull A shed *M. bovis* in semen for only a short period (seven weeks intermittently, and testing for carrier status was negative 17 months after semen collection started). The impact of *M. bovis* positive bull in transmission of the infection is unknown. Only few semen lots from bull A, and not all straws from these lots, tested positive. The high Ct values indicated low levels of the pathogen in the positive straws. *Mycoplasma bovis* growth was observed only in a 10^{-3} dilution. This suggests that the extender antibiotics have only a bacteriostatic but not a bactericidal effect. With bacteriostatic effect, it still has the potential to replicate and cause disease under the right conditions.

Several types of antibiotic have been added to seminal extenders before freezing to control bacterial contamination in semen. European Union (EU) directive 88/407 (EC, 1993) sets out the volume of antibiotics to be added to extended semen as follows: 500 IU per ml streptomycin, 500 IU per ml penicillin, 150 µg per ml lincomycin and 300 µg per ml spectinomycin. A so-termed GTLS mixture containing 500 µg gentamycin, 100 µg tylosin, 300 µg lincomycin and 600 µg spectinomycin per ml of extender without glycerol was developed by Shin et al. (1988) and is shown to be more efficient against mycoplasmas than a penicillin-streptomycin-polymyxin B combination. However, a later study by Visser et al. (1999) showed that a GTLS antibiotic combination has a bacteriostatic but not a bactericidal effect on different *M. bovis* strains used *in vitro* to contaminate semen. Recently Gloria et al. (2014) showed that a GTLS mixture failed to eliminate bacteria in frozen-thawed semen of one bull out of ten examined. Because mycoplasmas lack a cell wall they are intrinsically resistant to ß-lactam antibiotics. Several authors have reported an increase in the minimum inhibition concentrations (MIC) against several antimicrobial groups in European *M. bovis* isolates (Ayling et al. 2014; Gautier-Bouchardon et al. 2014; Heuvelink et al. 2016). In some of the studies

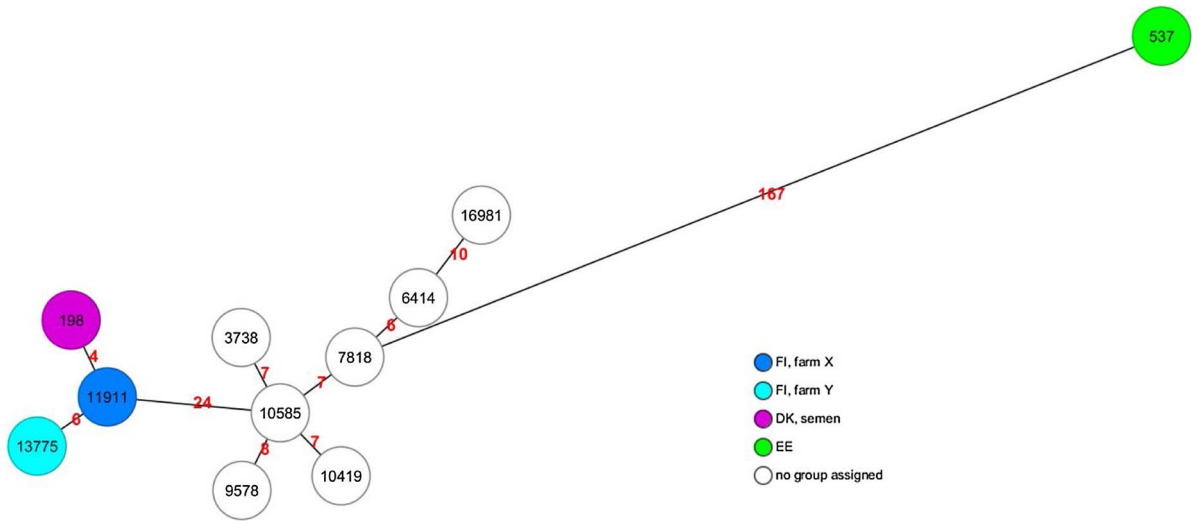

Fig. 3. Minimum spanning tree constructed from the cgMLST allele profiles. Allele differences between isolates are shown in red.

MIC values against tylosin and lincomycin / spectinomycin were quite high, thus the antibiotics used in semen extenders should be re-evaluated to ensure *M. bovis*-free semen or tested *M. bovis*-free semen should be available.

Several methods, mainly MLST and MLVA, have been used to genotype *M. bovis*. Molecular epidemiological studies report loss of diversity within *M. bovis* isolates over recent decades (Becker et al. 2015). Lately more detailed WGS-based methods targeting the whole genome are applied in characterization of mycoplasmas in epidemiological studies (Diaz et al. 2017), but only one conduct on *M. bovis* (Parker et al. 2016). Using whole genome SNP analysis, Parker et al. (2016) showed limited diversity among Australian *M. bovis* strains: there was a maximum of 50 SNP difference among 75 isolates collected during eight years. Similarly in our core genome MLST analysis, the Finnish strains isolated during four years clustered together and apart from an Estonian strain, indicating limited diversity among Finnish strains.

5. Conclusion

Our results indicate that *M. bovis*-positive semen used in insemination was the source of infection in two dairy farms, causing *M. bovis* mastitis. The infection source was identified by epidemiological analysis and cgMLST analysis of *M. bovis* isolates. Even though introduction of *M. bovis* into a herd via semen appears to be rare, semen has to be taken into account as a source of infection, and precautions need to be taken, especially in areas free of *M. bovis*, as well as in high-biosecurity herds. Global trade in semen may spread *M. bovis* to a new country or area. The antibiotics used in semen extenders should be re-evaluated to obtain *M. bovis*-free semen or tested *M. bovis*-free semen should be available.

Conflicts of interest statement

Of the authors, Henri Simonen works for Viking Genetics, an AI company owned by farmers from Denmark, Sweden, and Finland. This does not represent a conflict of interest to the scientific integrity of this article.

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